REMARKS

The Office Action dated August 4, 2006 has been received and carefully studied.

The Examiner maintains the rejection of claims 1, 7-10, 13-15, 18, 19 and 21, and newly rejects claim 22, under 35 U.S.C. §103(a) as being unpatentable over Aberg I, WO 98/56381 or Aberg II, WO 98/43640 in view of Polivka I or Polivka II.

The rejection is respectfully traversed.

The Examiner states that the biological system is well recognized to maintain stereo selectivity in its process, and cites references attempting to support her position. However, Applicants respectfully submit that the cited references do not support the Examiner's position. EMBASE 1985022681 (Cundy et al.) discloses evidence of *in vivo* metabolism of the R-isomer of nicotine, but not the S-isomer. Thus, this reference shows that administration of the racemic compound results in metabolism of only the R-isomer under the experimental conditions. It does not support the Examiner's statement that stereo selectivity is maintained during metabolism. EMBASE 1986195020(Bourgeois et al.) discloses that the metabolism of the R-isomer of MHT results in a different compound than the metabolism of the S-isomer of MHT. It also does not address the Examiner's position that stereo selectivity is maintained during metabolism. EMBASE 1991318244 (Boos et al.) discloses the urinary excretion of the optical isomers of unmetabolized ifosfamide. It is disclosed that

after administration of ifosfamide, more R-ifosfamide than S-ifosfamide was excreted, which according to the authors indicates stereoselective metabolism. This publication also does not disclose the maintenance of stereo selectivity of an optical isomer during metabolism.

Even if the cited references had been supportive of the Examiner's position, there are numerous instances where inversion or racemization during metabolism does occur. Indeed, Applicants submit that racemization during metabolism is the most common form of racemization in the body and the Racemases Enzyme Family in the human body is a very large and very active family of enzymes. These comprise hundreds of racemases and epimerases, which are catalyzing the racemization or inversion of a huge variety of metabolic events. Those skilled in metabolism know that the Racemases Enzyme Family belong to the Co-enzyme A Transferase Superfamily of enzymes. The racemization of enantiomeric drugs and the risk of formation of active metabolites *in vivo* is a major concern to all pharmacologists and chemists who are developing drugs containing optically active molecules.

Well-known examples of metabolic racemization include the metabolic racemization of amino acids (hundreds of publications exist, including Kochar et al., "Mechanisms of Racemization of Amino Acids by Aspartate Aminotransfersase", Eur J Biochem 1992, 203: 563-569, which discusses racemization in the liver, causing

L-alanin to become D-alanin, L-glutamate to become D-glutamate, etc.; Cloos, PA et al., Biochem J 2000, 345: 473-480, which discusses collagen fragment molecules undergoing racemization in the body to form four isomers; and Konishi T., et al., "In Vivo Studies on Chiral Inversion and Amino Acid Conjugation of 2-[4-(3-Methyl-2-thienyl)phenyl]propionic Acid in Rats and Dogs", Drug Metab Dispos 1999, 27: 158-160, which discloses the chiral inversion of R-2-[4-(3-Methyl-2-thienyl)phenyl]propionic acid in rats and dogs by enzymes belonging to the Co-enzyme A Superfamily of enzymes. Tang et al. described the inversion of (R)-stiripentol to (S)-stiripentol, but not (S)-to (R)-stiripentol (Tang C. et al., Metabolic chiral inversion of stiripentol in the rat. II. Influence of route of administration. Drug Metab Dispos. 1994, 22: 554 - 560). One of the inventors of the present invention has described the inversion of (R)- to (S)-ketotifen in eight animal species (Aberg et al Inversion of (R)- to (S)-ketoprofen in eight animal species. CHIRALITY, 1995, 7: 383-387). In collaboration with Jamali, this inventor also has described inversion of R- to S-ketoprofen as well as S- to R-ketoprofen in one and the same species (Jamali, F. et al. Bi-directional chiral inversion of ketoprofen in CD-1 mice. Chirality 1997, 9: 29-31).

As further evidence of the complete unpredictability of maintenance of stereo selectivity during metabolism, submitted herewith is a Declaration of Professor Thomas Walle, Medical University of South Carolina. Professor Walle avers that he has

personal experience from studies on the metabolism of ketotifen and ketotifen analogs to norketotifen, and that it is completely unpredictable whether the metabolism of atropisomerically pure S-ketotifen would lead to atropisomerically pure S-norketotifen.

Accordingly, one skilled in the art would have no reasonable expectation that administration of S-ketotifen would stereoselectively metabolize into S-norketotifen. Applicants again point out that the very existence of the atropisomer S-norketotifen was unknown prior to this invention.

The Examiner states that Attorney argued that the metabolite has less side effects than the parent Ketotifen, and that such argument has little probative value without support by factual evidence. The Examiner also states that no evidence or comparative value has been provided as to whether when the substantially pure S-metabolite of the claims was administered independently of the parent drug, there would be any difference in side effects. Applicants wish to remind the Examiner of the Declaration filed in July of 2003, which objectively demonstrates that surprisingly, S-norketotifen is free of sedative side effects, and that these data were in comparison to ketotifen, S-ketotifen, R-ketotifen, norketotifen and R-norketotifen. Furthermore, the Declaration also objectively demonstrates that surprisingly, S-norketotifen has less antimuscarinic side effects that norketotifen and R-norketotifen, while S-norketotifen is more active as an anti-inflammatory agent than norketotifen, which in

turn is more active than ketotifen. Accordingly, the arguments presented do have significant probative value, and rebut any *prima facie* case of obviousness that may have been established.

The Examiner cites page 3 of the specification for the statement that almost all of the sedative side effects were found to reside in R-ketotifen. However, this statement does not mean that S-ketotifen was devoid of sedative side effects. Indeed, the 2003 Declaration of record objectively demonstrates that although almost all of the sedative side effects reside in the R-isomer of ketotifen, the S-isomer of Ketotifen still exhibits sedative side effects. The specification recognizes that S-ketotifen does not have the severe sedative side effects of Ketotifen, but it does not state that the sedative side effects of S-ketotifen are "not an issue" as the Examiner asserts.

Nowhere is there any disclosure or suggestion in the cited art that the S-isomer of norketotifen actually exists or that this molecule is devoid of the severe sedative side effects of ketotifen. This alone is so surprising and unexpected that it rebuts any *prima facie* case of obviousness. Nowhere is there any disclosure or suggestion in the cited art that contrary to ketotifen, the S-isomer of norketotifen in reality is devoid of any clinically detectable antimuscarinic side effects. This also is surprising and unexpected and rebuts any *prima facie* case of obviousness.

The allowance of claim 6 is noted with appreciation.

Reconsideration and allowance of all pending claims are respectfully requested in view of the foregoing.

Respectfully submitted,

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ARTICLES

Mechanism of racemization of amino acids by aspartate aminotransferase

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Aspartate aminotransferase (mitochondrial isoenzyme from chicken) has been found to racemize very slowly dicarboxylic amino acid substrates in the presence of their cognate oxo acids [Kochhar, S. & Christen, P. (1988) Eur. J. Biochem. 175, 433-438]. Tyrosine, phenylalanine and alanine are racemized at the same rate although they undergo the transamination reaction 3-5 orders of magnitude more slowly than the dicarboxylic substrates. Similarly, the truncated enzyme aspartate aminotransferase-(27/32-410) catalyzes the racemization at the same rate as the native enzyme, while its rate of transamination is decreased to 3% of that of the native enzyme. Apparently, the rate- limiting step in racemization is not immediately linked to the transamination cycle. Decreasing the water concentration in the reaction medium by adding methanol at 0 degrees C drastically reduces the rate of racemization without affecting the rate of transamination. On the basis of these and additional kinetic data and the model of the three-dimensional structure of the active site, we conclude that a water molecule is responsible for the protonation of C alpha of the coenzyme-substrate intermediate from the wrong side. The diffusion of the water molecule into the interior of the enzyme appears to be the rate-limiting step in aspartate-aminotransferase-catalyzed racemization.

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Biochem. J. (2000) 345 (473–480) (Printed in Great Britain)

Collagen fragments in urine derived from bone resorption are highly racemized and isomerized: a biological clock of protein aging with clinical potential

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Abbreviations used: aL, native peptide; bL, isomerized peptide containing a b-Asp bond; aD, native peptide containing a D-Asp residue; bD, isomerized peptide containing a D-Asp residue; RI, racemization and isomerization, CTx, molecule comprising the sequence AHDGGR; TMB, 3,3',5,5'-tetramethylbenzidine.

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Key words: biochemical markers, bone turnover, isomerization, racemization.

Fragments of the a1 C-terminal telopeptide of type I collagen containing the sequence AHDGGR[1209–1214] (CTx) can be measured in urine as an index of bone resorption. We report here that these molecules undergo racemization and isomerization of Asp[1211] *in vitro* and *in vivo*, generating a mixture of four isomers: the native peptide form (aL), an isomerized form containing a b-Asp bond (bL), a racemized form containing a D-Asp residue (aD) and an isomerized/racemized form (bD). To study these reactions at this specific site in collagen, we have employed four immunoassays, each specific for one of the isoforms, and developed HPLC methods for their separation. The kinetics of these reactions were studied *in vitro* under physiological conditions by incubation of synthetic AHDGGR hexapeptide or mineralized bone collagen. Reactions were found to be strongly shifted towards the b-Asp forms and slightly in favour of the D-enantiomeric forms. CTx isomers were measured in human urine and in enzymic digests of bovine bone collagen. The results indicated that the extent of racemization and isomerization were correlated with the age and turnover of collagen. The ratios between the native and age-related forms of CTx were elevated in urine from patients with Paget's disease or osteoporosis as compared with that from healthy adults. The aL/aD CTx ratio had the highest discriminatory power (*T*-score = 23.2; $P < 0.0001$ and *T*-score = 1.5; $P < 0.0001$ for Paget's disease and osteoporosis respectively). In conclusion, these findings indicate that an assessment of CTx ratios in urine may provide an estimate of bone turnover, aiding in the diagnosis of metabolic bone diseases.

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Short Communication

In Vivo Studies on Chiral Inversion and Amino Acid Conjugation of 2-[4-(3-Methyl-2-thienyl)phenyl]propionic Acid in Rats and Dogs

(Received May 26, 1998; accepted August 7, 1998)

This paper is available online at http://www.dmd.org

ABSTRACT:

The relationship between chiral inversion and stereoselective amino acid conjugation of a new nonsteroidal anti-inflammatory agent, *R,S*-2-[4-(3-methyl-2-thienyl)phenyl]propionic acid (*R,S*-MTPPA) was investigated in rats and dogs. Only the *S*-enantiomer was found in plasma after oral administration of *S*-MTPPA to both species. In contrast, the *R*- and *S*-enantiomers were both detected after the dosing of *R*-MTPPA. In rats, the area under the curve of *S*-MTPPA in plasma was only 9% of that of *R*-MTPPA when *R*-MTPPA was dosed, whereas in dogs it was 2.5 times larger than that of the *R*-enantiomer. After administration of *R*-MTPPA, both enantiomers appeared in the urine. In rats, a small amount of *S*-enantiomer was found in the urine, whereas in the case of dogs the amount of the *S*-enantiomer was larger than that of the *R*-enantiomer. It appears that *R*-MTPPA is chirally inverted to *S*-MTPPA in both rats and dogs, although the extent of chiral inversion is greater in dogs than in rats. In dogs, the taurine conjugate was detected in plasma, urine and feces as a major metabolite after oral administration of either *R*- or *S*-MTPPA. In this case, only *S*-MTPPA-taurine was detected in the urine after the administration of *S*-MTPPA, and it was also the main component after administration of *R*-MTPPA.

R,S-2-[4-(3-Methyl-2-thienyl)phenyl]propionic acid (*R,S*-MTPPA),[1] a 2-arylpropionic acid derivative, is a new, orally effective, nonsteroidal anti-inflammatory agent. The *S*-isomer is pharmacologically active, whereas the *R*-isomer is inactive in vitro. The biological activities of *S*-MTPPA (code: M-5011) in animal models have been reported (Murakami et al., 1996; Kataoka et al., 1997; Tobetto et al., 1997; Kido et al., 1998). In our previous article, the disposition of *S*-MTPPA was studied after oral administration to rats, dogs, and monkeys using the ^{14}C-labeled drug. It was confirmed that the drug was metabolized mainly by oxidation of the thiophenyl moiety in these animals and by glucuronidation of the carboxyl group in rats and monkeys. In contrast, a major urinary and fecal metabolite in dogs was identified as the taurine conjugate of *R,S*-MTPPA (*R,S*-MTPPA-TAU) by means of isolation followed by mass spectrometry and ^{1}H NMR analyses (Konishi et al., 1998a).

It is well known that enantiomers of 2-arylpropionic acid derivatives undergo chiral inversion from *R*- to *S*-isomer (Yamaguchi and Nakamura, 1987; Caldwell et al., 1988; Baillie et al., 1989; Shirley et al., 1995). This chiral inversion is general, although variations exist among species and drugs (Lee et al., 1985; Mayer et al., 1988; Muller et al., 1990). The *R*- and *S*-enantiomers of 2-arylpropionic acid derivatives are known to have different pharmaceutical activities. For example, the inflammatory activities of the *S*-enantiomers of ibuprofen, naproxen, carprofen, and fenoprofen are stronger than those of the *R*-enantiomers in vitro (Gaut et al., 1975; Adams et al., 1976; Buttinoni et al., 1983; Kean et al., 1989). Therefore, an understanding of the extent of chiral inversion of the drugs is needed for analysis of pharmaceutical activity and toxicity. The mechanism of chiral inversion of 2-arylpropionic acid derivatives is thought to involve the CoA thioester as an intermediate (Nakamura et al., 1981). The CoA thioester is also a common intermediate of amino acid conjugation of 2-arylpropionic acid derivatives (Hutt and Caldwell, 1990; Asami et al., 1995). In this paper, we report the relationship of chiral inversion and taurine conjugation of MTPPA after administration of *R*- or *S*-MTPPA to rats and dogs.

[1]Abbreviations used are: MTPPA, 2-[4-(3-methyl-2-thienyl)phenyl]propionic acid; MTPPA-TAU, 2-[4-(3-methyl-2-thienyl)phenyl]propionic acid taurine conjugate; MTPPA-CoA, 2-[4-(3-methyl-2-thienyl)phenyl]propionic acid CoA thioester; HPLC, high-performance liquid chromatography; AUC, area under the curve.

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Materials and Methods

Materials. The enantiomers of *R,S*-MTPPA and their taurine and glycine conjugates were prepared at the Maruho Kyoto Research Laboratory (Kyoto, Japan) as described in a previous article (Konishi et al., 1998a).

Animals and Drug Administration. Sprague-Dawley strain male rats, 7 to 8 weeks old (weighing 170–240 g), were purchased from Japan SLC Inc. (Shizuoka, Japan). Male beagle dogs, 7 to 9 months old (weighing 14.1–14.7 kg), were purchased from Shimidzu Jikkenzairyo Inc. (Kyoto, Japan), and were given pellet food (DS, Oriental Yeast Co. Ltd., Tokyo, Japan).

S- or *R*-MTPPA was suspended in 0.5% carboxymethylcellulose sodium salt solution at the concentration of 10 mg/5 ml/kg for dosing and was orally administered to rats. The dogs received oral administration of *S*- or *R*-MTPPA in the dose of 10 mg/kg in a capsule.

Determination of Enantiomers of *R,S*-MTPPA. Extraction of *R,S*-MTPPA from plasma and urine was carried out by solid-liquid extraction and liquid-liquid extraction with diethyl ether. One milliliter of plasma or urine was treated with 2 ml of 0.2 N HCl and applied to a Sep-Pak C18 column (Waters, Milford, CT), which had been pretreated with 10 ml of methanol and 10 ml of water. The column was washed with 3 ml each of water, 1% acetic acid, and

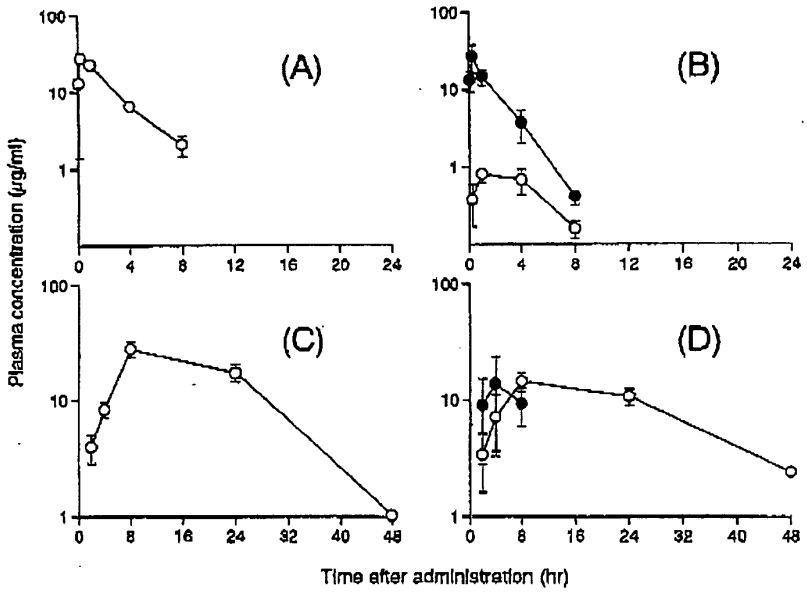

FIG. 1. *Plasma concentrations of S-MTPPA (○) and R-MTPPA (●) after oral administration of 10 mg/kg S-MTPPA* (A and C) *or R-MTPPA* (B and D) *to male rats* (A and B) *and male dogs* (C and D).

Each value represents the mean ± S.D. of five rats or three dogs.

1% acetic acid:methanol (6:4, v/v) in that order. Four ml of 1% acetic acid:methanol (1:9, v/v) was used to elute *R,S*-MTPPA. The eluate was evaporated under a nitrogen flow. The residue was taken up in 0.3 ml of 0.2 N HCl and 3 ml of diethyl ether, and the mixture was shaken for 10 min, and then centrifuged. The supernatant was evaporated to dryness and the residue was redissolved in 100 μl of ethanol. A 20-μl aliquot of this solution was injected into the high-performance liquid chromatography (HPLC) system according to the previously reported method (Konishi et al., 1998b).

Determination of *R,S*-MTPPA-TAU and its enantiomers. *R,S*-MTPPA-TAU from plasma, urine, and feces was determined according to the previously described method (Konishi et al., 1998a). In order to determine the enantiomers of *R,S*-MTPPA-TAU from urine, an aliquot of *R,S*-MTPPA-TAU extracted from the sample was subjected to the HPLC system according to the reported chiral direct separation method (Konishi et al., 1998b).

Results and Discussion

Plasma Concentration of *R*- and *S*-MTPPA in Rats and Dogs. Only the *S*-enantiomer was detected in plasma after oral administration of *S*-MTPPA to male rats. The area under the curve (AUC) of *S*-MTPPA was 88.5 μg·h/ml. In contrast, after administration of *R*-MTPPA, the *S*-enantiomer was detected in a small amount in addition to the unchanged *R*-enantiomer. The AUC of *S*-MTPPA after dosing of *R*-MTPPA was 5.1 μg·h/ml, which corresponds to 9% of that of unchanged *R*-MPTTA (56.0 μg·h/ml). When the plasma concentrations of unchanged enantiomer after administration of *R*- and *S*-MTPPA were compared, the C_{max} values were almost the same at about 27 μg/ml. However, the $T_{1/2}$ values were different, being 1.3 and 2.0 h, respectively (Fig. 1). The plasma concentrations of *R*- and *S*-enantiomers after oral administration of *R*- or *S*-MTPPA to male dogs are also shown in Fig. 1. Only the unchanged *S*-enantiomer was detected at each time interval after dosing of *S*-MTPPA. In contrast, a large amount of the *S*-enantiomer was detected in plasma after administration of *R*-MTPPA. The plasma concentration of the *S*-enantiomer at 8 h after dosing of *R*-MTPPA reached a peak, C_{max}, of 14.5 μg/ml. In this case, AUC of *S*-MTPPA was 397.4 μg·h/ml, which corresponds to 2.5 times that of the *R*-enantiomer.

Urinary Excretion of *R*- and *S*-MTPPA in Rats and Dogs. The *S*- and *R*-enantiomer were both excreted in urine until 24 h after oral administration of *S*-MTPPA to male rats. The urinary excretion of the *R*- and *S*-enantiomer was 0.07% and 3.2% of the dose, respectively. After administration of *R*-MTPPA, *R*- and *S*-enantiomer was also detected in the urine, and the amount was 0.7% and 0.2% of the dose, respectively. The excretion of unchanged *S*-enantiomer after administration of *S*-MTPPA was five times that of unchanged *R*-enantiomer after administration of *R*-MTPPA. The excretion of the *S*-enantiomer was 0.7% of the dose, but there was no *R*-enantiomer in dog urine after administration of *S*-MTPPA. When *R*-MTPPA was administered to dogs, the *S*-enantiomer (0.6%) was excreted in urine, in a higher amount than the unchanged *R*-enantiomer (0.2%).

It was detected *S*-MTPPA in plasma and urine after oral administration of *R*-MTPPA to rats. Conversely, a small amount of the *R*-MTPPA was detected in urine after dosing of *S*-MTPPA. The result shows that the inversion of *S*- to *R*-MTPPA also occurs in rats to a small extent, besides the *R*- to *S*-inversion. Chiral inversion of *R*-MTPPA to the *S*-enantiomer seems to be faster in dogs than in rats. Tanaka et al. (1992) reported that both *R*- to *S*- and *S*- to *R*-inversion of 2-phenylpropionic acid takes place in dogs. In this study, MTPPA was confirmed to show *R*- to *S*-inversion in dogs.

Amino Acid Conjugates of *R,S*-MTPPA. Glycine and taurine conjugates were assayed in urine and feces after administration of *R*- or *S*-MTPPA to rats and dogs. The taurine conjugate of *R,S*-MTPPA was detected in dog urine and feces after administration of *S*-MTPPA in amounts corresponding to 10.9 and 13.5% of the dose, respectively (Table 1). The taurine conjugate was also detected in dog urine and feces after administration of *R*-MTPPA in similar amounts to those found after dosing of *S*-MTPPA. In the urine of only one rat, the taurine conjugate was excreted after administration of *R*-MTPPA, in an amount corresponding to 0.6% of the dose. The glycine conjugate was not detected in any of the samples from rats or dogs (data not shown).

TABLE 1

Excretion of taurine conjugate of R,S-MTPPA in urine and feces for 48 h after oral administration of S-MTPPA or R-MTPPA at a dose of 10 mg/kg to male rats and dogs

Species	Administered Compound	Excretion of Taurine Conjugate (% of dose)	
		Urine	Feces
Dog	*S*-MTPPA	10.9 ± 1.9	13.5 ± 1.0
	R-MTPPA	13.5 ± 1.8	13.8 ± 1.6
Rat	*S*-MTPPA	ND[a]	ND
	R-MTPPA	0.6[b]	ND

Values are the mean ± S.D. of three animals.
[a] ND, not detected.
[b] Value for one animal.

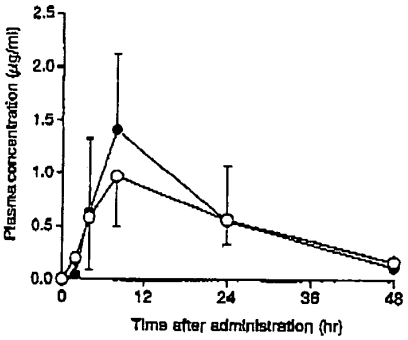

FIG. 2. *Plasma concentrations of R,S-MTPPA-TAU after oral administration of S-MTPPA (○) or R-MTPPA (●) at a dose of 10 mg/kg to male dogs (n = 3).*

Each value represents the mean ± S.D.

The plasma concentration of *R,S*-MTPPA-TAU after administration of each enantiomer of MTPPA to male dogs is shown in Fig. 2. The plasma concentrations after administration of either *R*- or *S*-MTPPA peaked at 8 h after administration. The C_{max} of *R,S*-MTPPA-TAU after the administration of *R*-MTPPA was 1.41 μg/ml higher than that after the administration of *S*-MTPPA (0.97 μg/ml).

Stereoselectivity of taurine conjugation of *R,S*-MTPPA in dogs was investigated by chiral HPLC separation of the enantiomers of *R,S*-MTPPA-TAU. After administration of *S*-MTPPA, only the *S*-enantiomer of the taurine conjugate (*S*-MTPPA-TAU) was detected in dog urine for 48 h, in an amount corresponding to 10.3 ± 2.7% (mean ± S.D. of four experiments) of the dose. In the case of *R*-MTPPA administration, *S*-MTPPA-TAU was mainly excreted, in an amount corresponding to 13.2 ± 2.0% of the dose, and the *R*-enantiomer of the conjugate was also detected in a small amount (0.5 ± 0.3%).

It was considered that *R*- and *S*-MTPPA are each transformed to the thioester, *R*- and *S*-MTPPA-CoA, which are then interinverted by epimerase as described for ibuprofen-CoA (Shieh and Chen, 1993). This may be followed by hydrolysis to *R*- or *S*-MTPPA or by conjugation with taurine to afford *R*- or *S*-MTPPA-TAU, respectively. It appears that the *S*-enantiomer does not convert to the *S*-enantiomer-CoA ester in rats, so presumably the *S*-enantiomer is not a suitable substrate for rat acyl-CoA synthetase (Fournel and Caldwell, 1986). However, in dogs, *S*-MTPPA is converted to *S*-MTPPA-CoA, as indicated by the appearance of *S*-MTPPA-TAU in the urine after administration of *S*-MTPPA. *R*-MTPPA-CoA was considered to be converted to *R*-MTPPA-TAU to a small extent and *S*-MTPPA was converted to *S*-MTPPA-TAU mainly via the *S*-MTPPA-CoA, because *S*-MTPPA-TAU was found mainly in the urine after administration of *R*- or *S*-MTPPA to dogs. Taurine *N*-acyl transferase may be selective for *S*-enantiomer-CoA, rather than *R*-enantiomer-CoA.

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Metabolic chiral inversion of stiripentol in the rat. II. Influence of route of administration.

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As described in the accompanying study, it was found that when the S enantiomer of stiripentol [(S)-STP] was given orally to rats, blood specimens contained only (S)-STP, whereas following administration of an equivalent dose of (R)-STP, both R and S forms of the drug were detected in the systemic circulation. In the present study, we investigated the influence of route of administration on this apparently unidirectional chiral inversion of (R)-STP in the rat. When (R)-STP was given either intravenously (60 mg kg-1) or intraperitoneally (300 mg kg-1), the inversion phenomenon was not observed, indicating that the process must take place presystemically. Following oral administration of either enantiomer of STP, it was found that the drug present at various points along the gastrointestinal tract became progressively enriched in molecules of R configuration, such that the free STP in cecum, large intestine, and feces consisted largely of the R enantiomer, regardless of the configuration of the administered drug. In a parallel in vitro study, it was demonstrated that STP undergoes acid-catalyzed racemization, the rate of which is appreciable at the pH value of the rat stomach (pH approximately 4). On the basis of these observations, it is proposed that the apparent metabolic chiral inversion of (R)-STP results from the combination of at least two factors: 1) partial acid-catalyzed racemization in gastric acid (that affects both enantiomers equally), and 2) enantioselectivity in one or more of the processes involved in the absorption, first pass metabolism or biliary excretion of STP, such that the S isomer appears selectively in the systemic circulation, whereas the R enantiomer is eliminated preferentially in the feces.

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Inversion of (R)- to (S)-Ketoprofen in Eight Animal Species

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ABSTRACT The (R)-enantiomer of the NSAID ketoprofen was administered orally at 20 mg/kg to a series of 8 animal species. In all species, a highly significant degree of inversion occurred after 1 h which varied from 27% (gerbil) to 73% (dog) and persisted or increased in plasma samples obtained 3 h after drug administration. Although the (R)-enantiomer was inactive as an inhibitor of cyclooxygenase, the analgesic effects of that isomer was almost the same as the (S)-isomer in animal analgesic assays, following oral administration of the drugs to mice and rats. Taken together, the present results suggest that (R)-ketoprofen administered alone functioned primarily as a prodrug for (S)-ketoprofen under the experimental conditions of this study.

KEY WORDS: nonsteroidal antiinflammatory drugs, chiral inversion, analgesia

The 2-arylpropionic acid (2-APA) nonsteroidal antiinflammatory drugs (NSAIDs) are a group of chiral analgesic/antiinflammatory agents usually administered as racemates. However, this class of drugs exhibits very significant differences in the pharmacological, toxicological and pharmacokinetic properties between their isomeric forms as described by Wechter et al.,[1] Jamali et al.,[2] Hayball et al.,[3] and others. Interestingly, several of these compounds undergo inversions in vivo, where the (R)-isomers transform to their (S)-antipodes.[1] However this process seems both compound and species specific.[4] With ketoprofen (KTP) marked inversion has been found in the dog and rat[4,5] while only minimal effects have been found in the gerbil,[4] rabbit,[6] and man.[7] Little if any inversion has been reported to occur in the guinea pig.[4]

The initial purpose of the present investigation was to estimate the extent of inversion of KTP in several animal species using standardized experimental conditions. Subsequently, the relative analgesic effects of the two enantiomers were assessed in a species in which the degree of inversion was limited (mouse) and one in which it was extensive (rat).

In this communication, the term "inversion" refers to changes in plasma drug levels only.

MATERIALS AND METHODS

Animals, Drug Administration, and Plasma Sample Collection

Mice Male CD-1 mice, obtained from Charles River Laboratories (Wilmington, MA), were group housed in temperature- and humidity-controlled rooms maintained on a 12 h light/12 h dark cycle. Food and water were provided ad libitum. For the inversion studies, groups of mice were dosed orally by gavage with (R)- or (RS)-KTP, 20 mg/kg in 0.25% methylcellulose. The animals were sacrificed at 1, 3, or 8 h following drug administration, blood was taken by intracardiac puncture and collected in heparinized tubes. The samples were centrifuged, plasma obtained and frozen at −70°C.

Rats Male Sprague-Dawley rats from Charles River Laboratories, Wilmington, MA were group housed as described above. For the inversion studies, groups of rats were dosed orally by gavage with (R)- or (RS)-KTP, 20 mg/kg in 0.25% methylcellulose, and blood was drawn retroorbitally from the conscious animals at 1, 3, and 8 h. Plasma was prepared and frozen as described above.

Guinea pigs Male Hartley guinea pigs (290–370 g) from Charles River Laboratories, Newfield, NJ were housed as described above. The animals were dosed orally with (R)- or (RS)-KTP, 20 mg/kg in 0.25% methylcellulose or with vehicle alone. The animals were sacrificed at 1 or 3 h after drug or vehicle administration and blood was drawn by cardiac puncture. Plasma was prepared and frozen as described above.

Gerbils and hamsters Male Mongolian gerbils (40–55 g) and male Golden Syrian hamsters (100–130 g) from Tumble Brook Farms and Charles River, respectively, were housed and dosed orally with (R)-KTP, 20 mg/kg in 0.25% methylcellulose as described above. The animals were sacrified 1 or 3 h after drug administation, blood was drawn by intracardiac puncture, centrifuged, and plasma frozen at −70°C.

Rabbits Male rabbits (2.3–3.0 kg) were obtained from Hazelton Research Products, housed singly in cages as described above, and given food and water ad lib. The animals were dosed orally with (R)-KTP, 20 mg/kg in 0.25% methylcellulose. Blood was drawn from the ear vein 1 and 3 h after drug administration, plasma obtained and frozen at −70°C.

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Dogs Eight male beagle dogs (8–10 kg) were purchased from Summit Ridge Farms, housed singly in cages on a 12 h light/12 h dark cycle, and given food and water ad lib. The animals were dosed orally with 20 mg/kg (R)-KTP. Venous blood was drawn 1 and 3 h after the dosing, plasma obtained and frozen at −70°C.

Monkeys Six adult male cynomolgus monkeys (6.0–8.8 kg) from the Bowman Gray breeding colony were selected. Their health records since birth were assessed and a physical examination was performed by an experienced veterinarian before any animal was entered into the study. All animals were dosed (R)-KTP, 20 mg/kg, by nasogastric intubation under slight ketamine (15 mg/kg, im) sedation. Venous blood samples were drawn under renewed ketamine sedation at 1 and 3 h after drug dosing. Plasma was prepared and frozen at −70°C.

Drugs and Analytical Methodology

All investigational drug samples of (R)-, (S)-, and (RS)-KTP were synthesized by Sepracor, MA. The identity of R-KTP (lot # 478-70) was confirmed by IR and UV spectral analysis. Both spectra were consistent with the structure for KTP. The chemical and isomeric purity of each of these lots was determined by HPLC using a chiral stationary phase (CSP). The (R)-KTP lot was determined to contain 99.28% (R)-KTP and 0.72% (S)-KTP. The racemic KTP lot was commercial BP grade (Wycoff, South Haven, MI) and was determined to be 99.7% pure and to contain 50% (R)-KTP and 50% (S)-KTP. All other chemicals were reagent grade (with the exception of *n*-butylchloride; GC grade) and obtained either from Sepracor or from commercial sources.

Prior to analysis, KTP and added internal standard (naproxen, 100 ng) were extracted from 1-ml aliquots of acidified plasma (50 μl 50% phosphoric acid, v/v) into *n*-butyl chloride (5 ml). The extracts were evaporated to dryness under vacuum and the analytes derivatized to their respective (−)-(S)-α-methylbenzylamide diasteriomers using the procedure of Avgerinos and Hutt[8] with minor modifications. The purity of the methylbenzyl amine reagent was 98% (Aldrich Chemical Co.).

For analysis, a Hewlett-Packard 5890 Series II gas chromatograph equipped with a Hewlett-Packard HP-1 fused silica capillary (15 m × 0.2 mm i.d., 0.11 μm film thickness) was used. Temperature settings were injector and transfer line 300°C, oven 100 to 170°C at 10°C/min (2 min hold at 170°C) followed by 170 to 290°C at 20°C/min with a 1 min hold at 290°C. The carrier gas was helium set at 1.5 ml/min; the injection volume was 1 μl. The analytes were detected by selective ion monitoring (210 *m/z* ketoprofen; 185 *m/z* internal standard) with a 50 msec dwell time and the electrode multiplier set at 400 V relative to autotune. Average retention times for (S)-KTP and (R)-KTP, and naproxen were 13.3, 13.5, and 12.8 min, respectively.

A Hewlett-Packard 5790 MS Chem Station HP-UX was used for data analysis. The standard curves (low curve 5–50 ng/ml; high curve 50–500 ng/ml/isomer) were prepared by spiking plasma with ketoprofen racemate standard (Sigma Chemical Co.). Quantitation of samples was calculated from a weighted ($1/x^2$) regression of the peak ratio response (isomer/Int. Stad.) vs concentration (ng/ml/isomer). The lower limits of quantitation for each isomer, in the presence of its antipode, was 5 ng/ml plasma.

Enzyme Assays

Human HL-60 promyelocytes grown in culture were induced to differentiate with DMSO. Subsequently they were incubated at a concentration of 3×10^6 cell/ml for 15 min at 37°C in the presence of various concentrations of test drug. The cells were stimulated to release arachidonic acid by the addition of the calcium iontophore A23187 (5×10^{-6} *M*) for 15 min. The secreted PGE_2 (cyclooxygenase product) and LTB_4 (lipoxygenase product) in the external medium were assayed by EIA using a commercially available kit.

Analgenic Tests

Phenylquinone-induced writhing Male mice obtained from Charles River Laboratories and housed internally for at least 4 days were divided into groups of 10 for use in this study. Their weight at study initiation was 18–30 g. The animals were dosed orally with test drug suspended in 0.25% methylcellulose (10 ml/kg) followed 1 h later by the intraperitoneal injection of 0.25 ml of 0.02% aqueous solution of phenyl-*p*-benzoquinone. Body writhing was then ascertained on an all or none basis for 10 min.[9]

Rat gait assay Male Sprague-Dawley rats were obtained from Charles River Laboratories, allowed to acclimate internally for at least 4 days, and then fasted for 18 h. Animals weighing between 130 and 150 g were then injected with 0.25 ml of a 35% Brewer's yeast suspension into the plantar surface of the left hind paw. Three hours later the animals were orally dosed with the test article suspended in 0.25% methylcellulose (5 ml/kg) and tested for analgesia 60 and 180 min later according to the general method of Atkinson and Cowan.[10] Scoring was done according to the following scheme: 2.0, normal gait; 1.5, intermittent limping; 1.0, constant limping; 0.5, limping with occasional 3 legged gait; 0, continuous 3 legged gait.

RESULTS

The plasma levels of total KTP and the inversion of (R)-KTP to (S)-KTP, after oral administration of (R)-KTP, are shown in Table 1. The "total plasma level" is a calculated figure, obtained by adding the analyzed plasma levels of (S)-KTP and (R)-KTP. There were significant differences in plasma levels of total KTP between species. Thus the "observed C_{max}" (1 h after drug administration) was more than 100 times higher in the mouse than in the guinea pig. As shown in Table 2, there were also significant differences in plasma drug elimination rates between species.

All animal species inverted (R)-KTP to (S)-KTP. The gerbil inverted (R)-KTP to the lowest extent in this acute study, to 27 and 33% (after 1 and 3 h, respectively) while the inversion in the dog was 73% after 1 h and 92% after 3 h. Two different groups of animals could be distinguished with regard to their ability to invert (R)-KTP. The "extensive inverters" (dog, hamster, rat) inverted (R)-KTP to a level significantly above 50% and "limited inverters" (mouse, gerbil, guinea pig, rabbit, monkey) demonstrated an inversion of approximately 50% or less. The plasma levels of (R)-KTP, (S)-KTP, and of

TABLE 1. Plasma levels of total KTP (in μg/ml) and of (S)-KTP (as percent of total KTP) 1 and 3 h after oral dosing of (R)-KTP, 20 mg/kg, to 8 species of animals

		1 h			3 h	
Species	N	Total KTP	% (S)-KTP	N	Total KTP	% (S)-KTP
Mouse	8	52.8 ± 4.9	39.4 ± 1.1	8	29.0 ± 5.7	44.1 ± 2.8
Rat	8	16.1 ± 1.4	55.9 ± 1.9	8	13.4 ± 0.7	73.7 ± 2.4
Gerbil	8	21.0 ± 3.7	27.0 ± 1.5	8	2.15 ± 0.35	32.9 ± 2.8
Hamster	8	13.3 ± 1.5	65.7 ± 1.4	8	4.23 ± 0.67	80.8 ± 1.9
Guinea pig	12	0.42 ± 0.10	41.9 ± 3.3	7	0.04 ± 0.01	46.7 ± 5.2
Rabbit	8	12.0 ± 0.9	35.4 ± 2.4	7	7.23 ± 0.81	50.7 ± 1.7
Dog	7	26.4 ± 7.0	72.8 ± 2.8	8	16.9 ± 1.8	91.9 ± 3.3
Monkey	6	38.0 ± 8.2	32.6 ± 1.4	6	10.2 ± 1.6	45.4 ± 4.4

TABLE 2. Change in percent of plasma levels of total KTP and of (S)- and (R)-KTP between 1 and 3 h after oral administration of (R)-KTP, 20 mg/kg

		1–3 h		
		Change in plasma conc. (%)		
Species	N	Total KTP	(S)-KTP	(R)-KTP
Mouse	8	−45.1	−33.8	−52.5
Rat	8	−16.4	−13.1	−59.4
Gerbil	8	−89.7	−87.5	−90.5
Hamster	8	−68.2	−61.4	−81.0
Guinea pig	12	−91.5	−90.4	−92.1
Rabbit	8	−39.8	−13.0	−45.7
Dog	7	−35.9	−18.2	−84.4
Monkey	6	−73.2	−63.6	−78.1

TABLE 3. Plasma levels of total KTP (in μg/ml) and of (S)-KTP (as percent of total KTP) 1, 3, and 8 h after oral dosing of (RS)-KTP, 20 mg/kg, to mice and rats and (R)-KTP, 20 mg/kg, to rats

		1 h		3 h		8 h	
Species	N	Total KTP	% (S)-KTP	Total KTP	% (S)-KTP	Total KTP	% (S)-KTP
Mouse	10	41.1 ± 2.6	57.6 ± 1.9	21.4 ± 2.1	54.0 ± 1.1	4.02 ± 0.57	41.3 ± 2.3
Rat (RS)	10	26.7 ± 2.6	76.0 ± 0.9	15.4 ± 1.5	83.1 ± 2.0	19.7 ± 1.6	93.0 ± 0.8
Rat (R)	10	23.6 ± 2.9	54.0 ± 1.4	11.8 ± 0.6	70.3 ± 2.6	20.6 ± 1.4	89.4 ± 0.8

total KTP decreased over time at different rates among the species, but there was no clear tendency for the "limited" inverting species to eliminate plasma drug levels faster than the "extensive inverters."

Subsequently, the inversion of racemic KTP was studied in both "extensive" and "limited" invertering animals. The rat and the mouse were chosen as representatives for each of the two categories of animals. Table 3 shows the total plasma levels of KTP and plasma concentration of (S)-KTP (as percent of total KTP) at 1, 3, and 8 h after an oral dose of 20 mg/kg of racemic (RS-)KTP. The rat metabolized (R)-KTP into (S)-KTP also when the drug was given as the racemate, while in the mouse, the ratio (S)-KTP/total KTP actually decreased in the time period between 1 and 8 h after dosing.

To put the above data in perspective, the relative abilities of the two isomers of ketoprofen to inhibit cyclooxygenase was evaluated in an assay in which PGE_2 production was the end point. In this assay the (S)-isomer, whose IC_{50} was 0.4 n*M*, was 250 times more potent than the (R)-enantiomer (Fig. 1). The contamination of 0.72% (S)-KTP in our sample of (R)-KTP can explain all the cyclooxygenase activity seen with (R)-KTP. Conversely in a lipoxygenase assay in which LTB_4 formation was the end point, no effects on LTB_4 production were seen by either (R)-, (S)-, or (RS)-KTP in concentrations up to 10 μ*M* (data not shown).

The analgesic effects of the two isomers were assessed following oral administration to mice and rats. In mice the ability of the compounds to inhibit writhing induced by the intraperitoneal injection of phenylquinone was the parameter assessed. The analgesic results shown in Figure 2 indicate that essentially equivalent results were produced with the two dose regimens, although the efficacy was slightly less (ap-

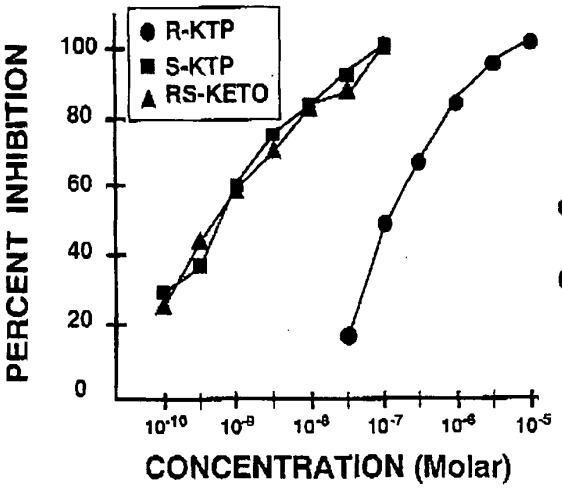

Fig. 1. Effects of (R)-, (S)-, and (RS)-ketoprofen on PGE_2 production of human granulocytes, stimulated by A23187. The calculated IC_{50} values, based on a line of best fit statistical method, were 0.4 n*M* for the RS and S forms of the compound and 100 n*M* for the (R)-isomer. SEM is < 10% of the mean values for all points shown in the figure.

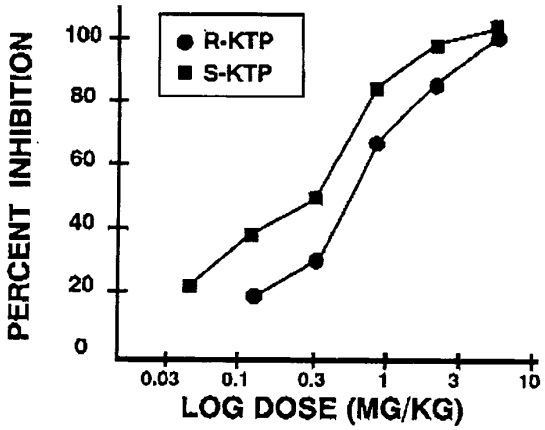

Fig. 2. Effects of (R)- and (S)-ketoprofen in the mouse writhing test. Each point represents the mean of 10 animals. SEM is < 10% of the mean values for all points shown in the figure. Test details are described under Materials and Methods.

proximately 1/3) in animals that had been given the (R)-isomer.

The "gait" test as originally described by Atkinson and Cowen[10] was employed in rats to assess analgesia. The results obtained 3 h after drug administration are shown in Figure 3 and indicate that the dose regimens were essentially equiactive in the rat. Minimally effective doses of each were in the 1–3 mg/kg range.

DISCUSSION

The plasma levels of total KTP varied greatly between species. Thus, the "observed C_{max}" (the highest observed

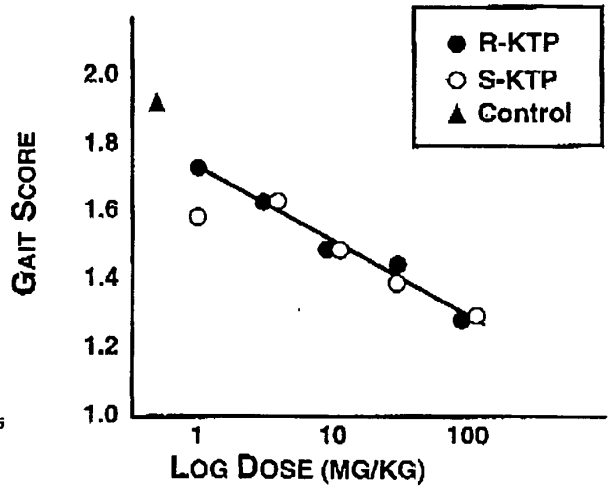

Fig. 3. Effects of (R)- and (S)-ketoprofen in rat gait assay. Each point is the mean of 10 animals. SEM is < 10% of the mean values for all points shown in the figure. All analgesic effects obtained for (R)- or (S)-ketoprofen at doses of 3 mg/kg or higher were significantly different ($P < 0.05$) from control using a Mann–Whitney *U* test.

plasma drug concentration, seen 1 h after drug administration) was 0.4 μg/ml in the guinea pig and 52 μg/ml in the mouse. This indicates significant variations in bioavailability and/or in plasma elimination rates. This study was not designed as a pharmacokinetic study, but it is obvious from Table 2 that there were very significant differences in plasma drug elimination rates among the species. Thus in the interval between 1 and 3 h after drug administration, the plasma level of total KTP decreased by approximately 90% in the guinea pig and the gerbil, but only by 16% in the rat. The extremely low plasma levels of KTP in guinea pigs may explain why KTP has a very low toxicity (LD_{50}, po, >1000 mg)[11] in that species. The acute toxicity is considerably higher in other species (LD_{50}, po, in the rat is approx. 150 mg/kg).[11]

The purpose of this study was to find an animal species that did not invert (R)-KTP and, therefore, could be used for toxicological studies of (R)-KTP. That goal was not reached since all the animal species inverted (R)-KTP to (S)-KTP, albeit to different extents and at different rates. As stated above, the gerbil demonstrated the lowest inversion of (R)-KTP and among the species that are commonly used for toxicological studies the observed inversions varied greatly: mouse 44%, rat 74%, rabbit 51%, dog 92%, and monkey 45% [all figures refer to ratio of plasma levels of (S)-KTP/total KTP, 3 h after dosing].

The animal species studied here can be divided into two different groups with regard to their ability to invert (R)-KTP. The animals belonging to the group called "extensive inverters" (dog, hamster, rat) inverted (R)-KTP to a level significantly over 50%, while the remaining species (mouse, gerbil, guinea pig, rabbit, monkey) demonstrated a 3 h inversion of (R)-KTP of 50% or less and were therefore denoted "limited inverters." As evident from Table 1, most of the inversion undoubtedly took place during the first hour after dosing, but Table 2 shows that even during the time interval from 1 to 3 h

after dosing, the plasma levels of (R)-KTP decreased 3–4 times faster than the plasma level of (S)-KTP in some of the species.

Although inversion of (R)-KTP to (S)-KTP is probably the main reason why certain animal species exhibit high plasma levels of (S)-KTP, enzymatic inversion may not be the only reason why an animal has unexpectedly high concentration of (S)-KTP in its bloodstream after dosing. Stereoselective absorption from the gut may be a factor when the racemate is dosed. The present findings, obtained under circumstances in which the (R)-enantiomer alone was administered, did not indicate stereoselective absorption to be of importance in rats or mice, which confirms the findings of Foster and Jamali[5] in the rat. Stereoselective biliary secretion and subsequent gastrointestinal reabsorption[5] and stereoselective renal excretion[11] may also influence the plasma levels of isomers. Thus in the rat, stereoselective gastrointestinal biliary secretion is the probable reason for the well-known second peak in plasma concentration in that species,[5] that is also evident in Table 3. The small decrease over time of the ratio (S)-KTP/total KTP that was seen in the mouse after administration of (RS)-KTP is interesting and may indicate stereoselective elimination of (S)-KTP or inversion of (S)-KTP to (R)-KTP in the mouse [and possibly in other species with "limited" inversion of (R)-KTP as well]. With the present data, we cannot provide an explanation why some species inverted (R)-KTP in the racemic drug differently from the pure isomer. The data from this study may indicate that (S)-KTP by some feedback mechanism inhibited the rate of enzymatic inversion of (R)-KTP in the "limited" inverters. Inversion of (S)-KTP to (R)-KTP has not been described, but is a possible explanation why the inversion was "limited" in certain species.

The biochemical mechanisms for the therapeutic effects of ketoprofen are considered to be due to inhibition of the enzyme cyclooxygenase.[11,13] In our inhibition assay of this enzyme, the (S)-isomer was clearly more active than the (R)-enantiomer. In fact, the potency difference was two orders of magnitude, confirming the results of others.[13] It is of course possible that optical impurities of the (S)-isomer were the reason for the weak cyclooxygenase inhibition seen in our tests with (R)-KTP.

In analgesic testing following oral administration to both rats and mice the two isomers appeared similar in potency, a finding which is in line with the conversion of the inactive (R)-enantiomer of the drug into the active (S)-isomer in these species. More precisely, in the rat where the results in the gait assay were identical for the two isomers, the degree of inversion of the (R)-isomer at the 3 h time point where the pharmacological measurements were made was 74% (see Table 1). On the other hand in the mouse writhing assay where the (S)-isomer was slightly more effective at each dose than the (R)-enantiomer the degree of inversion at the time point of the pharmacological assay (1 h) was only 39%.

It has been suggested that the analgesic activity of certain NSAIDs may not simply be a reflection of their ability to inhibit cyclooxygenase.[14] That is, other mechanisms particularly in the CNS, may be involved. Although our results do not refute these possibilities, the present results in animals may be adequately explained by the conversion of the (R)- into the (S)-enantiomer.

CONCLUSIONS

The present results demonstrate that (R)-ketoprofen is converted into its (S)-isomer in each of the 8 species studied. The degree of inversion however differs between the species with the dog showing the greatest and the gerbil the least degree of inversion. The present results also demonstrate the absolute necessity of using single isomers for all in vivo studies on enzymatic inversions in any species.

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Bi-directional chiral inversion of ketoprofen in CD-1 mice.

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The R enantiomers of some of the 2-arylpropionic acid non-steroidal antiinflammatory drugs (NSAIDs) are known to undergo metabolic chiral inversion to their more pharmacologically active antipodes. This process is drug and species dependent and usually unidirectional. The S to R chiral inversion, on the other hand, is rare and has been observed, in substantial extents, only for ibuprofen in guinea pigs and 2-phenylpropionic acid in dogs. After i.p. administration of single doses of racemic ketoprofen or its optically pure enantiomers to male CD-1 mice and subsequent study of the concentration time-course of the enantiomers, we noticed substantial chiral inversion in both directions. Following racemic doses, no stereoselectivity in the plasma-concentration time courses was observed. After dosing with optically pure enantiomer, the concentration of the administered enantiomer predominated during the absorption phase. During the terminal elimination phase, however, the enantiomers had the same concentrations. Our observation is suggestive of a rapid and reversible chiral inversion for ketoprofen enantiomers in mice.

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